

Exhibit C

<p>In re Application of:</p> <p>Gregory Hannon and Scott Hammond</p> <p>Serial No: 09/866,557</p> <p>Filed: May 24, 2001</p> <p>For: Methods and Compositions for RNA Interference</p>	<p>Art Unit: 1635</p> <p>Attorney Docket No. CSHL-P02-010</p> <p>Examiner: Sean McGarry</p>
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Declaration of Gregory Hannon and Scott Hammond under 37 CFR 1.131

Sir:

We, Gregory Hannon and Scott Hammond, state that we are the named inventors of the above-identified application and are the inventors of the subject matter claimed in the pending claims therein.

We understand that the Examiner has rejected certain claims of the pending application in view of the Li et al. published US Application 2002/0114784 (herein the "Li Application"), having an effective filing date of January 28, 2000. The claims of the above-referenced application, as presently amended, are directed to methods of using encoded hairpin RNA molecules that inhibit gene expression via an RNA interference mechanism. As evidenced *inter alia* by the exhibits attached hereto, we had possession of the claim invention before the January 28, 2000 effective filing date of the Li Application.

1. As described in further detail below, our laboratory generated hairpin RNA constructs for gene silencing before the filing date of the Li Application. However, we believe it is important to also understand that it was necessary to know the mechanism by which RNA interference ("RNAi") works in order to appreciate that hairpin RNAs could be used to induce gene silencing in mammalian cells. That is, in order for those skilled in the art to reasonably believe that a hairpin RNA could induce gene silencing, they first needed to understand the cellular mechanism by which double stranded RNA could induce sequence-specific gene silencing. As detailed below, at the time of the filing of the Li Application, that mechanism was not known to the public nor described in the Li Application. Moreover, the Li Application is entirely silent on the mechanism of RNAi. However, by that time (January 2000), our laboratory had

discovered the critical components of the RNAi mechanism necessary for reasonably predicting the utility of hairpin RNA for gene silencing.

2. At the time the Li Application was filed in January 2000, procedures based on double stranded RNA-triggered silencing were fairly well-established tools for functional genomics of lower organisms (plants, invertebrates and fungi). The ability of a few molecules of double stranded RNA to eliminate a much larger pool of endogenous mRNA had suggested a catalytic or amplification component to the interference mechanism. For instance, some of the plant literature favored an RNA-based copying system that was proposed to produce copious amounts of antisense RNA (while perhaps also producing additional sense and dsRNA). See Jorgensen et al. (1998) Science 279: 1486; Waterhouse et al. (1998) Proc. Natl. Acad. Sci. 95:13959 and Wassenegger et al. (1998) Plant Mol. Biol. 37:349.

If that had indeed been the mechanism, it would not be apparent how hairpin RNA could substitute for double stranded RNA.

3. At the time the Li Application was filed, one could certainly have hoped that RNA-triggered silencing would exist in vertebrates. However, the simple protocols used for invertebrate and plant systems were known not to be effective in mammalian cells. At that time, it was recognized in the art that there were several impediments to the use of RNAi in mammalian cells. Most mammalian cells harbor a potent antiviral response that is triggered by the presence of dsRNA viral replication intermediates. Reviewed Williams (1997). Biochem. Soc. Trans. 25, 509-513 and Gil (2000). Apoptosis 5, 107-114. In somatic cells, dsRNA activates a variety of responses. Predominant among these is PKR, a kinase that is activated by dimerization in the presence of dsRNA (Clarke et al. (1995) RNA 1, 7-20). PKR, in turn, phosphorylates EIF2 α , causing a nonspecific translational shutdown. dsRNA also activates 2'-5' oligoadenylate polymerase, the product of which is an essential cofactor for a nonspecific ribonuclease, RNase L. Reviewed in Baglioni et al. (1983). Interferon 5, 23-42. The ultimate outcome of this set of responses is cell death via apoptosis.
4. The recapitulation of the essential features of RNAi was a prerequisite for a biochemical analysis of the phenomenon. As a result of biochemical and genetic approaches our laboratory used in several experimental systems, the mechanisms underlying RNAi in both invertebrates and vertebrates began to unfold to the point that, by January 2000, we had recognized the utility of hairpin RNA for inducing gene silencing by an RNAi pathway. In particular, we identified the existence of conserved machinery for double stranded RNA-induced gene silencing from drosophila to mammals. We also defined the RNAi process as

proceeding via a two-step mechanism. In the first step, double stranded RNA is recognized by an RNase III family nuclease called Dicer, which cleaves the dsRNA into about 21-23-nt siRNAs (now called “small interfering RNA” or “siRNA” in the scientific literature). These siRNAs are incorporated into a multicomponent nuclease complex, RISC, which identifies substrates through their homology to siRNAs and targets these cognate mRNAs for destruction.

5. Exhibit A shows the results of an experiment in which S2 cells were transfected with dsRNAs corresponding to either cyclin E or lacZ.
 - i. Cellular extracts were incubated with synthetic mRNAs of lacZ or cyclin E. Extracts prepared from cells transfected with the cyclin E dsRNA efficiently degraded the cyclin E transcript; however, the lacZ transcript was stable in these lysates (see panel A). Conversely, lysates from cells transfected with the lacZ dsRNA degraded the lacZ transcript but left the cyclin E mRNA intact. These results indicate that RNAi can ablate target mRNAs in cultured cells through the generation of a sequence-specific nuclease activity. We termed this enzyme RISC (RNA-induced silencing complex).
 - ii. We observed that the nuclease activity present in the extract could also recognize the antisense strand of the cyclin E mRNA. Again, substrates that contained a substantial portion of the targeted region were degraded efficiently whereas those that contained a shorter stretch of homologous sequence were recognized inefficiently (See panel C, as600). For both the sense and antisense strands, transcripts that had no homology with the transfected dsRNA (panel B, Eout; Panel C, as300) were not degraded. Furthermore, the nuclease was inactive against a dsRNA identical to that used to provoke the RNAi response in vivo (panel B). In the in vitro system, neither a 5' cap nor a poly(A) tail was required, as such transcripts were degraded as efficiently as uncapped and non-polyadenylated RNAs.
 - iii. The figure provided in Exhibit A was received by the journal Nature on November 26, 1999 as part of a manuscript. The experiments described herein and the data used to create the figure of Exhibit A were carried out at my direction in the United States, prior to the January 28, 2000 filing date of the Li Application.
6. Gene silencing provoked by dsRNA is sequence specific. We reasoned that a plausible mechanism for determining specificity would be incorporation of nucleic-acid guide sequences into the complexes that accomplish silencing. In accord with this idea, pre-treatment of extracts with a Ca²⁺-dependent nuclease

(micrococcal nuclease) abolished the ability of these extracts to degrade cognate mRNAs (see data presented in Exhibit B). Activity could not be rescued by addition of non-specific RNAs such as yeast transfer RNA. As shown Exhibit B, although micrococcal nuclease can degrade both DNA and RNA, treatment of the extract with DNase I had no effect. Sequence-specific nuclease activity, however, did require protein. Together, our results support a mechanism in which the RNAi nuclease is a ribonucleoprotein, requiring both RNA and protein components. Biochemical fractionation (see below) is consistent with these components being associated in extract rather than being assembled on the target mRNA after its addition.

The figure provided in Exhibit B was received by the journal Nature on November 26, 1999 as part of a manuscript. The experiments described herein and the data used to create the figure of Exhibit B were carried out at my direction in the United States, prior to the January 28, 2000 filing date of the Li Application.

7. In plants, the phenomenon of co-suppression has been associated with the existence of small (25-nucleotide) RNAs that correspond to the gene that is being silenced. To address the possibility that a similar RNA might exist in *Drosophila* and guide the sequence-specific nuclease in the choice of substrate, we partially purified our activity through several fractionation steps. Crude extracts contained both sequence-specific nuclease activity and abundant, heterogeneous RNAs homologous to the transfected dsRNA (See Exhibit C). The RNAi nuclease fractionated with ribosomes in a high-speed centrifugation step. Activity could be extracted by treatment with high salt, and ribosomes could be removed by an additional centrifugation step. Chromatography of soluble nuclease over an anion-exchange column resulted in a discrete peak of activity (Exhibit C panel b, cyclin E). This retained specificity as it was inactive against a heterologous mRNA (Exhibit C panel b, lacZ). Active fractions also contained an RNA species of 25 nucleotides that is homologous to the cyclin E target (Exhibit C panel b, northern). The band observed on northern blots may represent a family of discrete RNAs because it could be detected with probes specific for both the sense and antisense cyclin E sequences and with probes derived from distinct segments of the dsRNA addition.

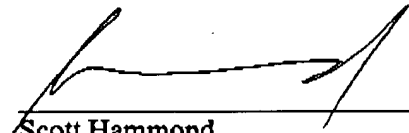
The figure provided in Exhibit C was received by the journal Nature on November 26, 1999 as part of a manuscript. The experiments described herein and the data used to create the figure of Exhibit C were carried out at my direction

in the United States, prior to the January 28, 2000 filing date of the Li Application.

8. RNA interference allows an adaptive defense against both exogenous and endogenous dsRNAs, providing something akin to a dsRNA immune response. Our data is consistent with a model in which dsRNAs present in a cell are converted, either through processing or replication, into small specificity determinants of discrete size in a manner analogous to antigen processing. Our results suggest that the post-transcriptional component of dsRNA-dependent gene silencing is accomplished by a sequence-specific nuclease that incorporates these small RNAs as guides that target specific messages based upon sequence recognition. The identical size of putative specificity determinants in plants and animals predicts a conservation of both the mechanisms and the components of dsRNA-induced, post-transcriptional gene silencing in diverse organisms. In plants, dsRNAs provoke not only post-transcriptional gene silencing but also chromatin remodeling and transcriptional repression. We made this argument in the manuscript that was received by the journal Nature on November 26, 1999, and therefore had possession of an understanding of the mechanism of RNAi prior to the January 28, 2000 filing date of the Li Application.
9. Exhibit D is a notebook page from the undersigned Scott Hammond's laboratory demonstrating the generation of a hairpin RNA construct corresponding to a LacZ sequence. The results of an experiment in which S2 cells were transfected with dsRNAs corresponding to either cyclin E or lacZ. The redacted date of this communication is prior to the January 28, 2000 filing date of the Li Application.

The undersigned hereby declare that all statements made herein are of their own knowledge and are true, and all statements made on information and beliefs are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issuing thereon.

Greg Hannon
Date:



Scott Hammond
Date: 3/5/07